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Need for an accurate molecular diagnosis to assess the donor origin of leukemia relapse after allogeneic stem cell transplantation

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ABSTRACT

Background and Objectives. **Leukemia relapse occurring in donor cells after allogeneic hematopoietic stem cell transplantation has been reported in rare cases. Cytogenetic analysis and molecular probing of variable number of tandem repeats (VNTRs) have been used to confirm this unusual event in the few cases so far reported in the literature. The aim of this study was to demonstrate that extensive molecular characterization of leukemic cells at diagnosis and relapse may be necessary to avoid many technical pitfalls possibly leading to an erroneous diagnosis of leukemia relapse in donor cells after allogeneic transplantation.**

Design and Methods**. We report the case of a 49-year old man who received an allogeneic transplantation from his HLA-identical sister because of BCR-ABL$^+$ acute lymphoblastic leukemia (ALL). After having achieved complete hematologic and molecular remission, two years later an overt leukemia relapse occurred with cytogenetic findings suggesting a leukemia relapse in donor cells. The donor or patient origin of leukemic cells at relapse was further investigated by fluorescence *in situ* hybridization (FISH) karyotyping, reverse transcription (RT) polymerase chain reaction (PCR) analysis of BCR-ABL chimeric transcripts, PCR amplification of several VNTRs and the Y chromosome-specific DYS14 sequence and finally by amplification, cloning and sequencing of the CDRIII region of the immunoglobulin heavy chain (IgH) gene.**

Results. **At the time of relapse, conventional and FISH karyotyping revealed the presence of a Ph1+ chromosome and a female karyotype in all the 25 metaphases analyzed and PCR amplification of the Y chromosome-specific DYS14 sequence was negative. Moreover, the molecular evaluation of hematopoietic chimerism performed by the NZ-22 VNTR allowed us to demonstrate that at the time of relapse, a consistent proportion of hematopoietic cells was of donor origin. However, the molecular cloning and sequencing of the CDRIII region of the immunoglobuin heavy chain (IgH) gene rearrangement in leukemic blasts at diagnosis and relapse demonstrated their identity thus formally proving the patient origin of both leukemic clones.**

Interpretation and Conclusions. **While the simplest interpretation of the apparent female karyotype at relapse is the consequence of a loss of the Y chromosome which in leukemic blasts took place along with duplication of an X-chromosome, this case strongly emphasizes the need for accurate and extensive molecular characterization to prove the donor origin of a leukemia relapse after allogeneic transplantation.**

Key words: alloBMT, donor cell leukemia, IgH gene rearrangement

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The occurrence of leukemic transformation of donor cells *in vivo* after allogeneic transplantation has been reported in several cases.[1] To date the pathogenic mechanisms, which most likely take place in the donor after engraftment, still remain to be elucidated and different hypotheses have been proposed. Possible environmental factors, transfer of oncogenic material from host to donor[2] and viral infections, such as that caused by Epstein-Barr virus (EBV),[3] have all been suggested to play a crucial role in generating the new transformation event. Interestingly, recent data have provided new evidence for a direct link between viral genes and specific chromosome translocations such as in the case of adenovirus E1A for EWS-FLI1 fusion transcript characteristic of Ewing's tumor.[4] However, at least in some cases the donor origin of the leukemia relapse could be misinterpreted because of the lack of adequate molecular analysis. The case we describe here underlines this possibility and strongly suggests that appropriate experiments and controls should be performed before such a diagnosis is made.

Design and Methods

Patient and donor samples

Peripheral blood or bone marrow samples were taken from the patient and the donor after informed consent, using forms approved by the Institutional Review Board, had been obtained.

Immunophenotyping

The immunophenotyping was performed by flow cytometry on freshly isolated leukemic cells using the following fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies: anti-CD45, CD13, CD33, CD3, CD5, CD7, CD19, CD20, CD10, CD34, HLA-DR, CD11b and FACScan analyzer (Becton Dickinson, San José, CA, USA).

Karyotyping

Conventional karyotyping of leukemic blasts was performed according to standard methods of G-banded chromosome preparations on freshly isolated cells and after *in vitro* incubation for 48 hours with and without mitogens. Fluorescence *in situ* hybridization (FISH) was performed using commercially available BCR-ABL double color labeled probes (Oncor, Gaithersburg, MD, USA).

Molecular findings

Total cellular RNA and high molecular weight DNA were extracted according to standard methods.[5] Reverse transcription-polymerase chain reaction (RT-PCR) of the BCR-ABL chimeric transcript and amplification of variable number of tandem repeat (VNTR) loci including ApoB, ApoC2, YNZ-22, MCT118, 33.6, D11S533 or the Y chromosome specific DYS14 sequence were performed using a Thermal Cycler 9600 apparatus (Perkin Elmer Cetus, Norwalk, CT, USA) as described elsewhere.[5,6] PCR amplification, cloning and sequencing of the CDRIII region of the IgH gene rearrangement were performed as previously described.[5]

Results

Case report

In October 1996 a 49-year old man was admitted to our Department with a diagnosis of acute lymphoblastic leukemia (ALL). His hemoglobin concentration was 10.7 g/dL, white cell count 8×10^9/L, platelet count 25×10^9/L and his bone marrow was packed with lymphoblasts showing an immunophenotype consistent with a diagnosis of B-precursor ALL ($CD19^+$, $CD10^+$, $CD20^+$, $CD34^+$, HLA-DR^+, $CD33^-$, $CD11b^-$). While conventional cytogenetic analysis showed a normal karyotype, the molecular RT-PCR analysis of t(9;22) chromosome translocation documented a BCR-ABL gene rearrangement (e1a2 breakpoint) coding for the p190 chimeric oncoprotein. The patient was treated according to the institutional protocol and enrolled into an allogeneic stem cell transplantation program. In January 1997, after a conditioning regimen based on busulfan (16 mg/kg) and melphalan (110 mg/m^2) he received granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (5×10^6 $CD34^+$ cells/kg) collected from an HLA-identical female sibling donor. Cyclosporin A (CyA) and methotrexate were given for graft-versus-host disease (GVHD) prophylaxis. With this treatment, the patient achieved a complete hematologic and molecular remission as documented by repeated negative results of nested RT-PCR analysis of the BCR-ABL gene. Twenty-four months after transplantation, because of fever, myalgia and low back pain, bone marrow was aspirated and despite the lack of morphologically identifiable leukemic blasts, RT-PCR analysis documented the reappearance of the BCR-ABL (e1a2) chimeric transcript. Two months later a bone marrow aspirate revealed an overt leukemia relapse (more than 50% leukemic blasts) with an immunophenotype similar but not identical to that documented at diagnosis ($CD19^+$, $CD10^+$, $CD20^-$, $CD34^+$, HLA-DR^+, $CD33^+$, $CD11b^+$). Conventional cytogenetic analysis 47, XX, del(9) (p21), +i(17) (q10), del(20) [47, (q12), ider(22) (qter), t(9;22) (q34;q11)] revealed the presence of multiple karyotypic abnormalities including the Ph1+ chromosome and surprisingly, an apparent female karyotype in all the 25 metaphases analyzed (Figure 1, panel A). FISH analysis and RT-PCR confirmed the presence of the $Ph1^+$ chromosome and that of a BCR-ABL (e1a2) chimeric gene (Figure 1, Panel B and Figure 2, panel A). The patient did not respond to a further chemotherapy program and died soon after.

Molecular evaluation of the origin of the leukemic relapse

The discrepancy between a normal karyotype at diagnosis and the presence of a $Ph1^+$ chromosome at relapse could reflect the well known inability of cytogenetic analysis to detect the neoplastic clone in some cases of ALL. Most striking however, was the presence of an apparently female karyotype in leukemic blasts isolated at the moment of relapse which prompted us to confirm at the molecular level the donor origin of the leukemia relapse. Although PCR analysis had revealed the same BCR-ABL (e1a2) chimeric transcript in leukemic blasts at diagnosis and relapse, the huge length (> 55 Kb) of the minor breakpoint cluster region (m-bcr) did not allow us to perform a simple Southern blot analysis to investigate the DNA breakpoints of the BCR gene on chromosome 22.[7] Therefore we performed PCR amplification of the Y chromosome-specific DYS14 sequence in DNA samples isolated at diagnosis and relapse. As shown in Figure 2, panel B, while the male origin of the leukemic clone at diagnosis was evident, no

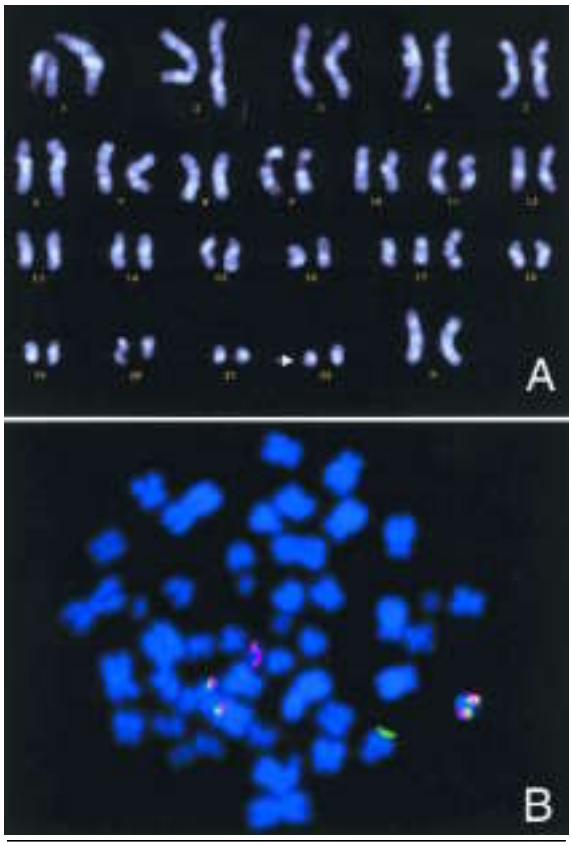

Figure 1. Karyotypic analysis at relapse. Conventional cytogenetic analysis of the leukemic blasts at relapse indicates an apparently female karyotype (panel A) and the presence of a Ph1+ chromosome (arrow). Fluorescence *in situ* hybridization using differently colored BCR and ABL probes allowed the detection of the Ph1+ chromosome (red, yellow and green spots), the abnormal chromosome 9 (red/green spots), the normal chromosome 22 (red spot) and 9 (green spot) (panel B).

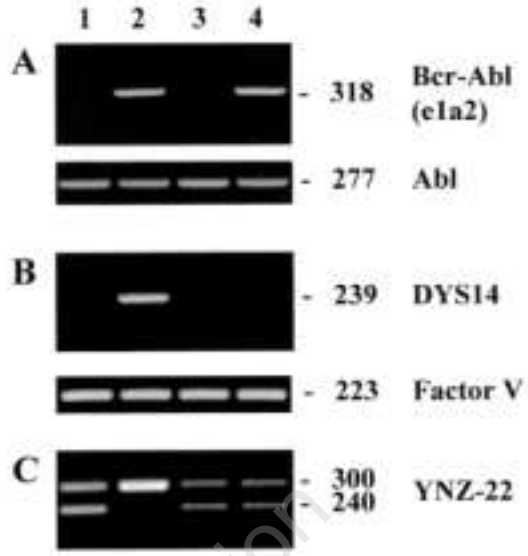

Figure 2. Molecular evaluation of donor and host origin of leukemic cells. Molecular analysis on RNA or DNA obtained from donor hematopoietic stem cells (lane 1), leukemic blasts at diagnosis (lane 2), patient's bone marrow at day +365 after transplantation (lane 3) and patient's bone marrow at relapse (lane 4). Panel A summarizes the RT-PCR analysis of the BCR-ABL chimeric transcript (e1a2 breakpoint) with the amplification of the normal ABL gene used as RNA quality control. Panel B and C report the PCR amplification of a Y-chromosome sequence (DYS14) and a chromosome 17 derived VNTR (YNZ-22). Amplification of the coagulation factor V gene was used as a control of the DNA quality.

amplification of the DYS14 sequence occurred at relapse. To identify other, more informative probes, the molecular analysis of several VNTRs including Apo B, Apo C2, MCT118, and 33.6, D11S533 was performed but none of these discriminated between patient and donor cells (data not shown). The analysis performed with the YNZ-22 probe, while suggesting that at the time of relapse a relevant proportion of hematopoietic cells were of donor origin, could not formally rule out the presence of patient's leukemic cells because of co-migration of two alleles (Figure 2, panel C). Therefore a further set of experiments was performed in order to identify an unequivocal marker of clonality. We then decided to clone and sequence the IgH gene rearrangements detected in leukemic blasts isolated at diagnosis and relapse. This set of experiments was performed by PCR amplification, cloning and sequencing of the CDRIII region of the IgH gene rearrangements.[5] As shown in Figure 3, the DNA sequencing demonstrated the identity of the two CDRIII regions thus formally proving the patient origin of the leukemia relapse.

Discussion

Here we report a leukemia relapse, occurring in a man after allogeneic transplantation from an HLA-identical sister, in which cytogenetic analysis strongly suggested the donor origin of the leukemic cells. This diagnosis was further suggested by the PCR results obtained using a sensitive Y-chromosome specific molecular probe and by the molecular analysis performed with the YNZ-22 VNTR.[6] However, the possibility of generating a leukemia-specific probe based on the unique DNA rearrangement of the IgH gene allowed unequivocal demonstration of the patient origin of the leukemic relapse. Because many additional, secondary genetic defects can occur after a myeloablative conditioning regimen, the simplest interpretation of the apparent female karyotype at relapse is the consequence of

Figure 3. Sequence analysis of the IgH gene rearrangement at diagnosis and relapse. Alignment of the DNA sequences of the CDRIII region obtained by amplification using VH and JH consensus primers (arrows). Boxed sequences correspond to the extra nucleotides (N) inserted at the junction between the D and J regions of the IgH gene.

the loss of the Y chromosome which could have eventually taken place along with duplication of an X-chromosome. Leukemia transformation of engrafted human bone marrow cells was first described more than thirty years ago[8] when the lack of adequate molecular tools at that time cut down the chances of making a precise molecular diagnosis of the leukemic clone. Therefore, in the majority of cases, conventional cytogenetics has been used to confirm the donor origin of the leukemic relapse and several technical pitfalls may have been encountered. More recently, sophisticated, FISH-based cytogenetic techniques have also been employed but usually they have been equally applied to investigate sex-related chromosomes.[1] Interestingly, the use of molecular probes for the evaluation of highly polymorphic DNA sequences (VNTRs) and restriction fragment length polymorphisms (RFLPs) in some cases corroborated, with more robust evidence, the diagnosis of leukemia recurrence in donor cells.[2] In other cases, however, this molecular analysis allowed correction of the erroneous assignment of a donor origin to a secondary leukemia thus confirming the inadequacy of a diagnosis based only on sex-related cytogenetic markers.[9] However, in some cases, it was shown that even the molecular analysis of VNTR could not definitively assign the origin of the leukemic clone because deletion or amplification of chromosomal segments carrying marker loci may lead to a misinterpretation of results.[10,11] Overall, only a few cases of those so far reported in the literature could provide an unequivocal and leukemia-specific molecular marker demonstrating the donor origin of the leukemia relapse after allogeneic transplantation. Our results strongly emphasize the need to identify leukemia-specific sequences, which may represent the only definitive tool to identify the origin of a malignant clone during its natural history in a patient despite possible further genetic evolution.

Contributions and Acknowledgments

OS contributed to the design of the study and carried out the molecular biology experiments. US and AM performed the cytogenetic analysis. GMB and ML helped in doing the molecular biology experiments. GD followed the patient clinically. TB is the head of the department and critically revised the manuscript. AR designed the study and wrote the manuscript.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- Our results indicate that a diagnosis of leukemia relapse in donor cells can not be supported only by cytogenetic evidence and must be demonstrated by an accurate molecular characterization of the leukemic cells using unequivocal, clone specific molecular probes. In the absence of such a characterization, the diagnosis of leukemia relapse in donor cells should not be accepted.

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